Analytical and Field Test Methods for Measuring BTEX Metabolite Occurrence and Transport in Groundwater

by

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PART I:

QUANTITATIVE DETERMINATION OF BENZYLSUCCINIC ACID BY SOLID PHASE EXTRACTION WITH IN-VIAL ELUTION

INTRODUCTION

Groundwater contamination due to leaky underground fuel tanks is a common problem at civilian and military sites worldwide. The U.S. EPA estimates that 35% of the U.S.'s underground motor fuel tanks are leaking (1), which corresponds to approximately 2 million tanks, and approximately 40% have resulted in groundwater contamination (2). Benzene, toluene, ethylbenzene, and xylenes (known collectively as BTEX) are water-soluble fuel constituents that comprise 50 wt % of the water-soluble fraction of gasoline (3). The occurrence of BTEX in groundwater is of concern due to the hazards that they pose toward human health. For example, benzene is a confirmed carcinogen and toluene, while it is less toxic than benzene, depresses the central nervous system. The EPA water quality criterion for benzene and toluene in drinking water is 0.005 mg/L and 14.3 mg/L, respectively (4).

Although BTEX is biodegradable under aerobic conditions (5), most groundwater at BTEX-contaminated sites is under anaerobic conditions. For this reason, the biodegradation of BTEX under anaerobic conditions has received much attention. Toluene degradation has been reported under anaerobic denitrifying (6-12), iron-reducing (13, 14), sulfate-reducing (11, 15, 16), and fermentative-methanogenic conditions (17, 18).

Because treatment of contaminated groundwater is expensive, it is important to know if biodegradation of contaminants, such as BTEX, is occurring naturally at a site prior to

undertaking a lengthy and costly treatment program. Currently, there are no direct methods for assessing whether intrinsic bioremediation is occurring at a particular site. Indirect methods such as measuring geochemical indicators, such as electron acceptor (e.g., oxygen, nitrate, iron, and sulfate) and their relation to contaminant spatial distributions currently are used (19); however, the results can be ambiguous. For this reason, a recent National Research Council report (20) recommended seeking BTEX metabolites that can be unambiguously related to anaerobic BTEX biodegradation. In addition, such metabolites ideally should have no commercial source and be chemically and biologically stable (20, 21). The detection of such metabolites would serve as ideal indicators of BTEX biodegradation under field conditions.

Novel anaerobic degradation products of BTEX including benzylsuccinic acid (BSA) and benzylfumaric acid were identified in anaerobic laboratory microcosm experiments under denitrifying (6-12) and sulfate-reducing conditions (11, 15, 16). Because these degradation products do not have a commercial source and are only produced during the biodegradation of toluene and *m*- and *o*-xylenes, they potentially fit the criteria as unambiguous indicators of *in situ* BTEX bioremediation (21). The proposed pathway for the formation of BSA and benzylfumaric acid was given by Evans et al. (10) (Figure 1).

Evans (10) proposed that the methyl group of toluene and *o*-xylenes is attacked by succinyl-coenzyme A, which is a strong nucleophile formed as part of the Krebs cycle, to form benzylsuccinic acid and, upon hydrolysis, benzylsuccinic acid forms benzylfumaric acid. Although BSA and benzylfumaric acid initially were hypothesized as biologically stable, recent reports indicate that they are intermediates in the degradation of toluene to benzoyl-CoA (11, 22). If BSA and benzylfumaric acid are transient intermediates, they may occur in groundwater

only at low concentrations. However, if their transient formation can be measured, such measurements could be used to determine the *in situ* rate of biodegradation.

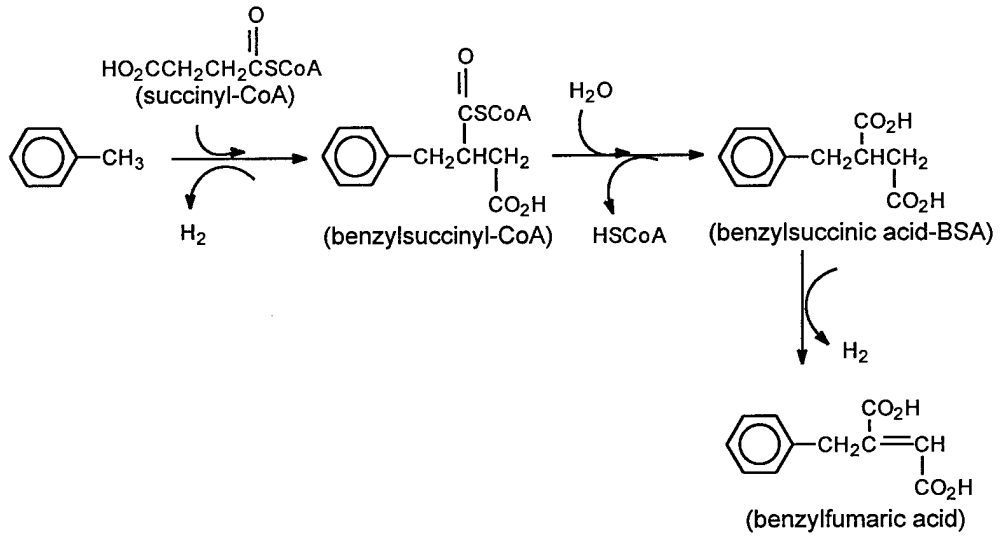

Figure 1. Proposed pathway for the formation of benzylsuccinic acid (BSA) and benzylfumaric acid from the nucleophilic attack by succinyl-CoA on toluene (from (10)).

Although the BSA and benzylfumaric acid metabolites of BTEX compounds were identified in laboratory experiments and during a field experiment in which toluene and xylenes were injected into an anaerobic BTEX-contaminated aquifer (21), to the best of our knowledge, nothing has been reported on the transport behavior *per se* of the metabolites in groundwater. As part of a larger research effort aimed at developing a simple field test to determine the *in situ* rates of BTEX biodegradation under anaerobic conditions, it is important to characterize the transport properties of BTEX metabolites.

Unfortunately, the analytical method used by Beller et al. (21), which consists of acidifying the sample to pH < 1 followed by liquid-liquid extraction is multi-step and

cumbersome and therefore, not conducive to the analysis of large numbers of field samples. In addition, derivatization with diazomethane is hazardous because the precursor of diazomethane is carcinogenic and the process of diazomethane generation potentially is explosive. For these reasons, an alternative extraction and derivatization method was desirable prior to undertaking field experiments.

Solid phase extraction is an attractive alternative to liquid-liquid extraction because it reduces the time, number of handling steps, and amount of organic solvent waste generated compared to liquid-liquid extraction methods. For solid phase extraction, cartridge and membrane (flexible disk) formats currently are available with a selection of sorbent phases from which to choose. The flexible disk format is composed of 8 μm particles embedded in a Teflon membrane, which eliminates any bed channeling because the particles are fixed. In addition, sample filtration and extraction are coupled into a single step, which reduces sample handling. Furthermore, the higher cross-sectional area of the disks compared to extraction cartridges allows for higher sample flow rates that decrease analysis times.

Organic acids such as BSA are very water soluble and therefore are not expected to efficiently partition into hydrophobic sorbents such as C18-bonded phase silica. In contrast, anion exchange sorbents are selective for the isolation of acids and therefore have a higher capacity for the acids than hydrophobic sorbents.

Compared to solid phase extraction cartridges where analytes must be eluted and physically separated from the cartridge, flexible extraction disks can be placed directly into small (2 mL) autosampler vials. This process, termed "in-vial" elution, is rapid, simple, and uses less organic solvents. Analytes that do not require derivatization, such as the herbicide diuron, can

simply be eluted from hydrophobic sorbents by placing the disk directly into organic solvent (23). For analytes that required derivatization, such as BSA, SAX disks are placed in an autosampler vial containing an alkylation reagent (methyl iodide). When heated, the acid analyte simultaneously elutes from the SAX disk, which acts as a catalyst, and is derivatized to its methyl ester. In our laboratory, SAX disks were employed for the extraction of several other acidic classes of compounds including the metabolites of the pesticide Dacthal in water and soil (24, 25); chlorophenoxy acid herbicides in surface water (26), and the acid metabolites of nonionic surfactants in municipal sewage effluent, paper mill effluent, and river water (27).

For this study, the first objective was to develop and validate solid phase extraction with in-vial elution as a quantitative, rapid, simple, and cost-effective alternative analytical method for the determination of BTEX metabolites in groundwater. The second objective was to use the developed analytical method to analyze samples obtained during field experiments aimed at determining the *in situ* transport behavior of BSA in groundwater.

EXPERIMENTAL METHODS

Reagents and Standards. Standards of benzylsuccinic acid (BSA; 99% purity) and 4-fluorobenzoic acid (4FBA; 98 % purity) were purchased from Sigma Chemical Co. (St. Louis, MO). Standards of 2-chlorolepidine (2-chloro-4-methyl quinoline; 99% purity) and methyl iodide were obtained from Aldrich Chemical Co. (Milwaukee, WI). For this study, 4FBA was selected as the surrogate standard because it was used as a surrogate standard by Beller et al. (21) and because it does not occur in gasoline. The 2-chlorolepidine was added as an internal standard in order to provide a measurement of the absolute recovery of BSA and 4FBA.

Acetonitrile (HPLC-grade) was obtained from Fisher Scientific (Fairlawn, NJ), acetone and methanol were obtained from EM Science (Gibbstown, NJ), and anhydrous ethyl ether was obtained from Mallinckrodt (Paris, KY).

Stock solutions of 1 μg/μL BSA, 4FBA, and 2-chlorolepidine were prepared in acetonitrile. Lower concentrations (0.100 μg/μL and 0.010 μg/μL) of BSA stock solutions were made from dilutions of the 1 μg/μL stock into acetonitrile.

Samples. The groundwater samples used in this study were collected from groundwater monitoring wells located near the Oregon State University motorpool in a sandy aquifer, which is screened from 0.5 m below land surface to the bottom of the well. Monitoring Well 4, which is screened to a depth of 6.03 m, is located a few meters from the site of a leaking underground gasoline storage tank that has since been removed. Monitoring Well 4 groundwater is characterized by dissolved oxygen concentrations of 0.2 to 0.5 mg/L, a pH of 7.4, and a nitrate concentration of 0.4 mg/L. Monitoring Well 2, which is screened to a depth of 4.05 m, is located approximately 40 m from Monitoring Well 2 and the groundwater in that well is not contaminated with gasoline. Groundwater from Monitoring Well 2 is characterized by dissolved oxygen concentrations of 0.5 mg/L and a pH of 7.0; nitrate concentrations were not measured.

Prior to sampling each well, approximately 25 L of groundwater were removed by baling and discarded to ensure that all stagnant water from the well casing and sand pack was removed so that samples were representative of water within the aquifer. Groundwater was collected in 4L baked brown glass bottles with Teflon-lined lids. All samples were collected without filtration and stored at 4 °C until analysis.

Extraction and Derivatization Efficiency. Initially, it was desired to compare the extraction and reaction efficiency of the solid phase extraction and in-vial elution process to that of conventional liquid-liquid extraction coupled with diazomethane derivatization as described by Beller et al. (21). Unfortunately, methylated standards of BSA and 4FBA were not available commercially. Therefore, the first step involved creating standards of each analyte by what is termed the "direct diazomethane" process that entailed the reaction of BSA and 4FBA (in their free acid form) with the direct addition of diazomethane (e.g., no extraction is involved). From these standards, gas chromatographic responses were established, and calibration curves were created. Next, samples were extracted by a microscale liquid-liquid extraction with ethyl ether followed by derivatization with diazomethane that was patterned after the procedures described by Beller et al. (21). By comparing the recovery of BSA and 4FBA by microscale liquid-liquid extraction to the calibration curve constructed by the direct diazomethane process, the efficiency of the liquid-liquid extraction step could be determined. The final set of experiments focused on the preparation of samples by the solid phase extraction with in-vial elution process and the comparison of BSA and 4FBA recovery obtained to thta of liquid-liquid extraction.

Several criteria were used to determine and compare the extraction/reaction efficiency of the conventional liquid-liquid extraction and solid phase extraction with in-vial elution method. First, information was needed on the absolute recovery of BSA and 4FBA to determine if losses occurred during extraction; the recovery of BSA and 4FBA were obtained by comparing the peak area ratios to that of the internal standard, 2-chlorolepidine. Second, information on the recovery of BSA relative to 4FBA was needed to determine if 4FBA was a suitable surrogate for BSA; the recovery of BSA relative to 4FBA was determined by comparing the peak areas of BSA to those

of 4FBA. Once it was established that 4FBA was a suitable surrogate for BSA, 4FBA could be added to environmental samples in order to compensate for any losses of BSA during sample processing.

Stock solutions of methylated BSA and 4FBA were prepared by adding diazomethane directly to solutions of BSA and 4FBA, which were in their free acid forms. Diazomethane was prepared using procedure II, "Preparation of alcohol-free ethereal solutions of diazomethane", given by the Aldrich Chemical Company with the purchase of Diazald. Briefly, butyl carbitol (2-(2-ethoxy-ethoxy)-ethanol) and ether are added to a solution of potassium hydroxide in water. This solution is placed in a 100 mL long-necked distilling flask fitted with a dropping funnel and efficient condenser and placed in a water bath heated to 70 °C. As distillation of the ether begins, a solution of Diazald in ether is added through the dropping funnel and the flask occasionally is shaken; the ethereal distillate contains diazomethane. To 1.0 mL of 1 μg/μL BSA stock solution, approximately 1 mL of diazomethane was added until a bright yellow color persisted. Excess diazomethane and ether were removed under nitrogen evaporation until a volume of approximately 1 mL was obtained. Acetonitrile then was added for a final volume of 10 mL, which yielded 0.1 μg/μL solutions of BSA and 4FBA in their methylated forms.

From the stock solutions of methylated BSA and 4FBA, a series of dilutions were made to create calibration curves. In order to determine the absolute and relative recovery of BSA, dilutions were prepared to give a final volume of 1 mL acetonitrile by spiking 10 μL of 1 μg/μL 2-chlorolepidine, 100 μL of 0.1 μg/μL 4FBA, and from 5 to 500 μL of 0.1 μg/μL BSA in its methylated form. In order to determine the absolute recovery of 4FBA, a calibration curve was prepared from a series of dilutions made by combining from 1 to 50 μg 4FBA in its methylated

form and 10 μg 2-chlorolepidine. All samples were analyzed by gas chromatography/flame ionization detection (GC/FID).

The micro-scale liquid-liquid extractions were performed on small (6 mL) samples in 15 mL screw-cap glass centrifuge tubes by adding 4 mL of deionized water together with 1.6 mL of 1.2 M HCl (final pH 0.5), 500 μL of 1 μg/μL BSA, and 14 μL of 1 μg/μL 4FBA stock solution to give a final sample concentration of 83 mg/L BSA and 2.3 mg/L 4FBA. This solution simulated environmental samples preserved by acidification to pH 0.5, which is similar to that reported by Beller et al. (21). Triplicate extractions were performed by adding 3 mL of diethyl ether to each centrifuge tube and shaking for 2 minutes after which the ether was removed. The combined ether extracts were reduced to approximately 2 mL by evaporation under nitrogen and dried by passing the extract over a bed of granular sodium sulfate. Each extract was derivatized with approximately 2 mL of diazomethane; excess diazomethane and ether were removed by evaporation with nitrogen until a volume of approximately 1 mL was obtained. Approximately 2 mL of acetonitrile were added and the volume was reduced again to less than 2 mL under nitrogen evaporation. At this point it is assumed all diazomethane and ether were removed. To this final solution 50 μL of 1 μg/μL 2-chlorolepidine was spiked and the resultant mixture quantitatively transferred to a 5 mL volumetric flask. The extracts then were analyzed by GC/FID and GC/MS.

Small-scale solid phase extraction with in-vial elution experiments were performed to compare the efficiency of extraction and reaction to that of conventional liquid-liquid extraction with diazomethane derivatization. The approach was similar to that of Field and Monohan (24) and to the methodology described in full detail in the following section for environmental

samples. Briefly, 13 mm SAX disks were used to extract 1 to 50 μg of BSA and 10 μg of 4FBA that had been spiked into 40 mL samples of deionized water. A second set of samples was extracted in order to determine the absolute recovery of 4FBA by spiking 1 to 50 μg of 4FBA into 40 mL samples of deionized water. In-vial elution was carried out by adding 100 μL of neat methyl iodide and 10 μg of 2-chlorolepidine to the autosampler vial containing the SAX disk and heating for 1 hr at 80 °C. All samples were analyzed both by GC/FID and GC/MS.

Solid Phase Extraction. For environmental samples, 25 mm diameter SAX disks (Varian, Sugarland, TX) were placed in 25 mm screw-together polypropylene filter holders (Micro Filtration Systems, Dublin, CA) that were attached to a vacuum manifold (Supelco, Bellefonte, PA) and fitted with a 75 mL polypropylene reservoir (Figure 2). The disks were pre-conditioned with 4 mL of acetone, allowed to soak for 30 s, and then dried under vacuum at 20 mm Hg for 1 min. The disks then were prewet with 4 mL methanol and allowed to soak for 30 s; after adding the methanol, the disks were not allowed to dry. The disks then were washed with deionized water (2 x 10 mL) prior to the addition of sample.

Each 250 mL environmental sample was spiked with 5 μg of 4FBA and thoroughly mixed, carefully poured into the reservoir, and drawn through the disk under full vacuum (23 mm Hg). The sample bottle was rinsed with deionized water (3 x 10 mL), added to the reservoir, and drawn through the disk. Once the sample and sample bottle rinse water had passed through the disk, the reservoir walls were rinsed with deionized water (4 x 5 mL). To initiate the process of drying the disk, the reservoir was removed and 60 mL of air were pushed through the disk by attaching a small hand-held syringe to the top of the polypropylene disk holder. Lastly, the top portion of the disk holder was removed and the disk was dried under full vacuum for 60 minutes.

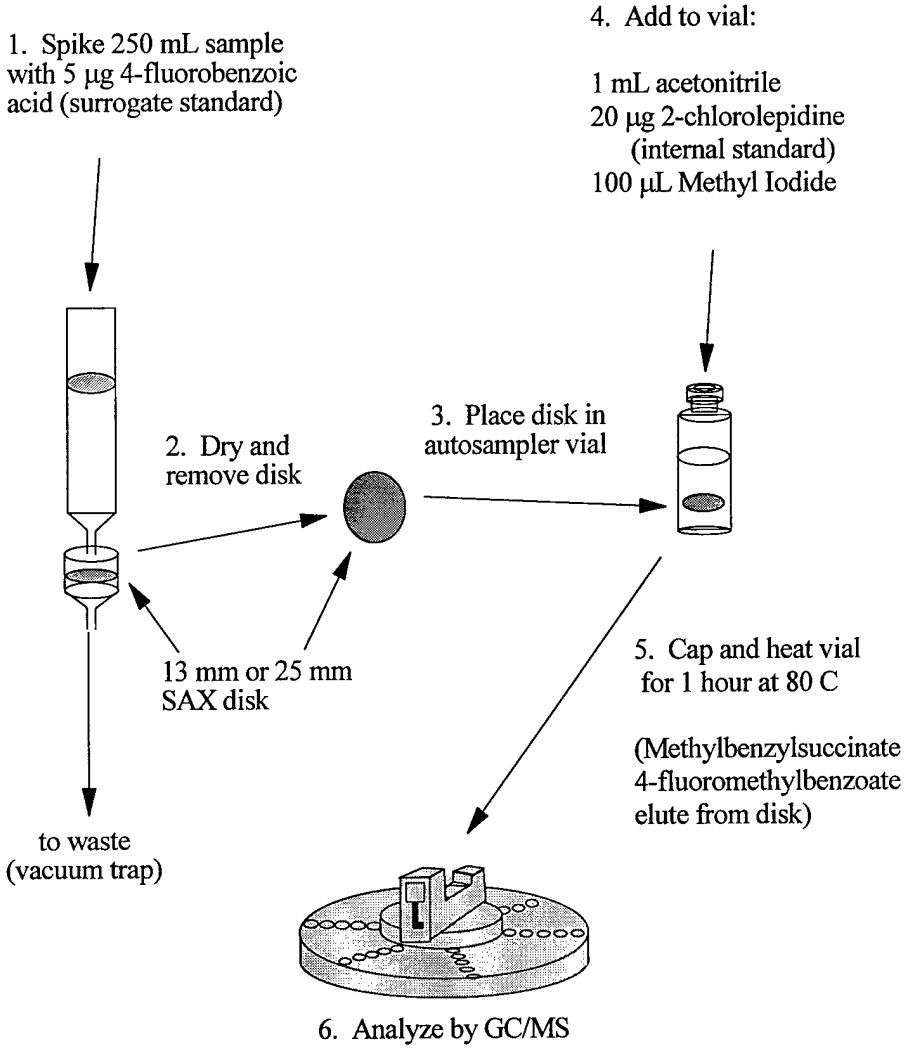

Figure 2. Schematic indicating steps in solid phase extraction with in-vial elution.

To perform in-vial derivatization and disk elution, the dried disk was placed in a 1.8 mL autosampler vial to which approximately 1.0 mL of acetonitrile, 20 µL of a 1 µg/µL solution of 2-chlorolepidine, and 200 µL of neat methyl iodide were added. The autosampler vial then was capped, shaken, and heated for 1 h at 80 °C. After cooling to room temperature, the samples

were analyzed by GC/MS.

Spike and Recovery. Spike and recovery experiments were performed with 250 mL samples of deionized water, uncontaminated groundwater collected from Monitoring Well 2 and gasoline-contaminated groundwater obtained from Monitoring Well 4. Groundwater collected from Monitoring Well 2 and 4 both were determined to have BSA concentrations below detection such that they could be used to determine the recovery of BSA from actual groundwater matrices. In addition, no benzylfumaric acid was detected in the samples. Five 250 mL samples of each sample matrix water were spiked to give a final concentration of 10 μg/L BSA and 20 μg/L 4FBA. The samples were extracted with the 25 mm SAX disk procedure as described above.

Chromatographic Analysis. All samples were analyzed using a Hewlett Packard Model 5890 Series II gas chromatograph equipped with a J & W DB-1 column (30m x 0.32 mm x 5 μm film thickness; J&W Scientific, Folsom, CA) and operated with helium as carrier gas. An injection volume of 1 μL was used under splitless conditions with an injector temperature of 250 °C. The initial oven temperature was held 1 min at 65 °C, ramped at 10 °C/min to 165 °C, followed by a 4 °C/min ramp to a final temperature of 265 °C. Note that such a thick film column is not required for analysis of the compounds in this study. The GC/MS was being used for analysis of highly volatile compounds related to a different project.

Mass spectral detection was performed with a Hewlett Packard 5972 mass selective detector (MSD) operated in electron impact ionization mode (70 eV) with an interface temperature of approximately 170 °C. The mass spectrometer was operated in both full scan (50-400 amu) and selected ion mode (SIM) using dwell time of 100 ms for each mass. For

quantitation in SIM mode, two ions were used to identify and quantify each analyte: BSA (m/z 176 and 236 [M^+]), 4FBA (m/z 123, 154 [M^+]), 2-chlorolepidine (m/z 142, 177 [M^+]). The ions m/z 202 and 234 ([M^+]) were selected to monitor for the presence of benzylfumaric acid. A m/z 234 was selected since it represents the [M^+] of the methylated form of benzylfumaric acid and is consistent with the loss of 2 hydrogen atoms from benzylsuccinic acid, which has [M^+] of m/z 236. An authentic standard of benzylfumaric acid was not available from which to identify a retention time nor confirm the ions needed to detect benzylfumaric acid. The ions representing the molecular ion of each analyte is denoted by the symbol [M^+].

Quantitation. Calibration curve samples were created by the 13 mm SAX procedure previously described. To determine the absolute and relative recovery of BSA, five-point calibration curves were constructed over BSA mass ranges from 1 to 10 μg and from 20 to 400 μg and each calibration standard contained 5 μg 4FBA and 20 μg 2-chlorolepidine as internal standard. The lower mass range was used to evaluate the spike and recovery experiments and the higher mass range was used to quantitate BSA in samples obtained during field push-pull tests. A third calibration curve was constructed to quantify samples containing a lower concentration of BSA; the BSA mass range for this calibration curve ranged from 0.2 to 5.0 μg BSA and each calibration sample contained 5 μg 4FBA and 5 μg 2-chlorolepidine as internal standard. A five-point calibration curve was constructed for determining the absolute recovery of 4FBA and ranged in 4FBA mass from 1 to 50 μg and each calibration standard contained 20 μg 2-chlorolepidine as internal standard.

RESULTS AND DISCUSSION

Extraction/Derivatization Evaluation. Since methylated standards of BSA and 4FBA were not available commercially, the initial phase of research focused on derivatizing methyl ester standards in order to create calibration curves from which the extraction/derivatization efficiency of conventional liquid-liquid extraction and solid phase extraction with in-vial elution could be judged. As expected the direct diazomethane methylated standards produced linear calibration curves with r^2 typically 1.000 as determined by GC/FID (Figure 3). The absolute recovery of BSA by micro-scale liquid-liquid extraction was 108.5%, which indicated that, as expected, the liquid-liquid extraction step is quantitative. In the GC/FID chromatograms a coeluting peak interfered with the quantitation of 4FBA so that its recovery could not be determined by GC/FID. High recovery of BSA was consistent with the recoveries of >70% reported by Beller et al. (21), which indicated that the calibration curve generated by the direct diazomethane process was valid for quantifying BSA in extraction samples.

In contrast to liquid-liquid extraction, the absolute recovery of BSA and 4FBA from samples processed by solid phase extraction with in-vial elution was significantly higher than 100% when calibration curves developed from standards generated by the direct addition of diazomethane were used for quantitation (Figure 3). The absolute recoveries of BSA and 4FBA were 133.8 ± 15.3% (11.4% RSD) and 121.3 ± 7.5% (6.2% RSD), respectively. The higher RSDs were probably due to the fact that only single samples of each standard were analyzed and averaged together so that the precision reflects variation over a range in BSA mass. The significantly higher recovery of BSA and 4FBA from the SAX disk indicates that reaction between BSA and 4FBA and methyl iodide is 20-30% more efficient than with diazomethane.

Although quantitative recovery was obtained with liquid-liquid extraction, the greater reaction efficiency of the solid phase extraction with in-vial elution method potentially will have better detection limits due to the increased signal to noise ratios at low BSA concentrations.

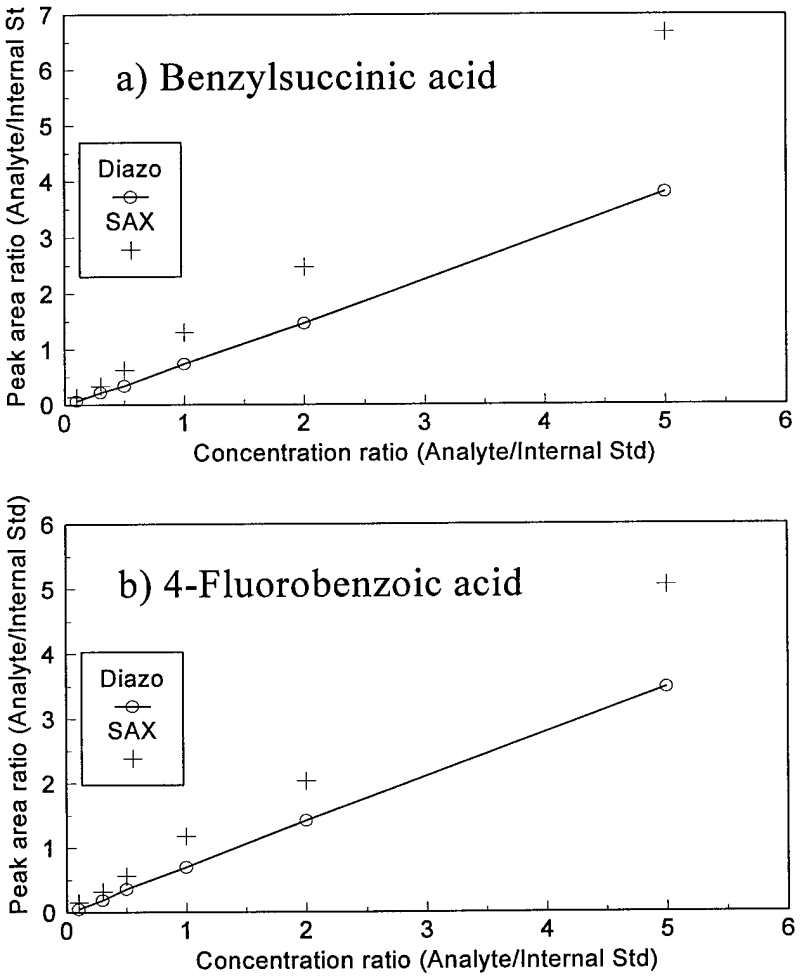

Figure 3. Responses (peak area ratio vs. concentration ratio) of (a) methylated benzylsuccinic acid (BSA) and (b) methylated 4-fluorobenzoic acid (4FBA) to methylation by reaction with diazomethane and to solid phase extraction with in-vial elution and derivatization with methyl iodide.

Although the greater reaction efficiency for BSA and 4FBA under in-vial elution conditions was greater than that achieved with diazomethane, the recovery of BSA relative to 4FBA was 98.3 ± 5.2% (5.3% RSD), which indicates that the reaction behavior of 4FBA is similar to that of BSA. For this reason, 4FBA is a good surrogate standard for BSA in the solid phase extraction with in-vial elution process.

Because the absolute recoveries greater than 100% were obtained from calibration curves based on reaction with diazomethane, and because these samples gave a linear calibration curve with r^2 typically 1.000, all subsequent calibration curves were created using the solid phase extraction with in-vial elution process. Linear calibration curves also were obtained when the calibration samples were analyzed by GC/MS. In addition, because environmental samples potentially contain interfering compounds, all subsequent analyses were performed by GC/MS.

Chromatograms obtained by GC/MS indicate good separation between 4FBA, BSA, and 2-chlorolepidine with retention times at 13.63, 28.81, and 27.51min, respectively (Figure 4a). The EI mass spectrum for BSA was similar to that reported in Beller et al. (21), including observation of the molecular ion [M]+ at 236 (Figure 4b), which indicates that the derivatization procedure produces the methylated forms of both BSA and 4FBA. The methyl ester of benzoic acid also was detected in full scan chromatograms (Figure 4a) at 14.11 min when only deionized water was passed through the disk, which indicates that benzoic acid is an artifact associated with the SAX disk and this finding was confirmed by 3M, the manufacturer of the SAX disks. Although benzoic acid does not interfere with the determination of BSA, 4FBA, or 2-chlorolepidine, it could represent an interference for some applications (e.g., the determination of benzoic acid in environmental samples). Although steps to remove the benzoic were not

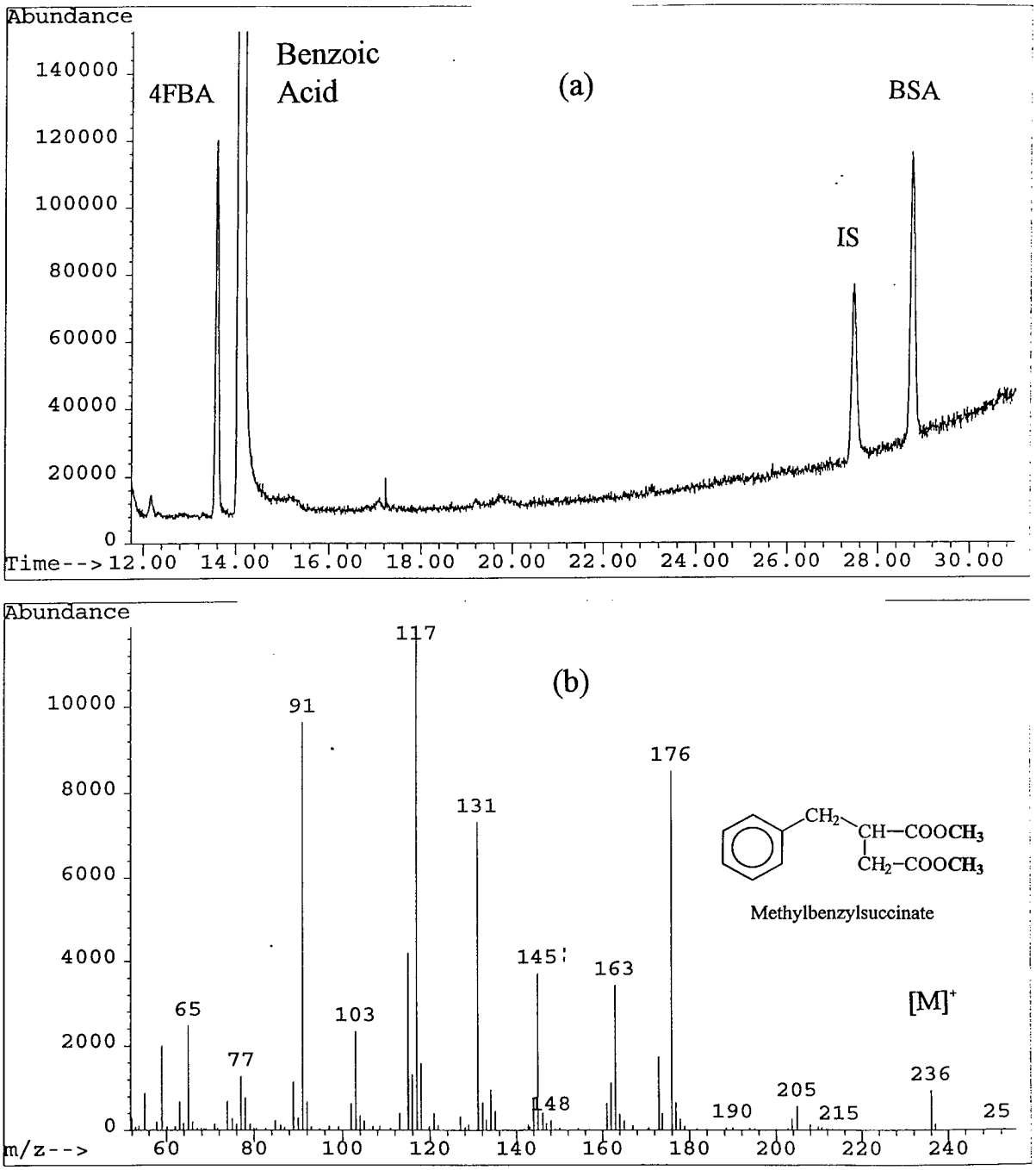

Figure 4. Full scan chromatogram of a standard containing BSA, 4FBA, and 2-chlorolepidine (a) and the EI-mass spectrum benzylsuccinic acid in its methylated form (b).

included as part of the method, SAX disks may be pre-soaked in 12 mM HCl/acetonitrile solution to remove the benzoic acid.

Accuracy and Precision. After establishing that the solid phase extraction with in-vial elution method was more efficient for derivatizing BSA compared to conventional liquid-liquid extraction coupled with diazomethane derivatization, it was necessary to verify that scaling-up from 40 mL samples and 13 mm SAX disks to larger sample volumes (e.g., 250 mL) would give similar recoveries of BSA and 4FBA. The average recoveries of BSA and 4FBA from five replicate 250 mL samples of deionized water extracted using 25 mm SAX disks were 86.2 ± 1.7% (2.0% RSD) and 86.5 ± 1.1% (1.3% RSD), respectively (Table 1). Nearly equivalent recoveries of BSA and 4FBA resulted in a high recovery of BSA relative to 4FBA (98.2 ± 2.8%, 2.8% RSD), which indicated that 4FBA remained a good surrogate standard for BSA. Consistently low RSDs indicate good precision for the larger samples.

Although compounds such as substituted naphthalenes were detected in full scan chromatograms obtained by GC/MS (Figure 5a) , presumably due to interaction with the styrene divinyl benzene polymer matrix of the SAX disk, they did not interfere with the quantitation of BSA, 4FBA, or 2-chlorolepidine. No BSA was found above detection when the unspiked groundwater from either Monitoring Well 2 or 4 was analyzed in SIM mode (Figure 5b). For this reason, groundwater from Monitoring Well 4 was used to determine BSA recovery from a gasoline-contaminated matrix. Typical chromatograms for the spiked gasoline-contaminated groundwater obtained under SIM acquisition indicated good signal to noise for BSA, 4FBA, and 2-chlorolepidine even in the gasoline-contaminated groundwater matrix (Figure 5c).

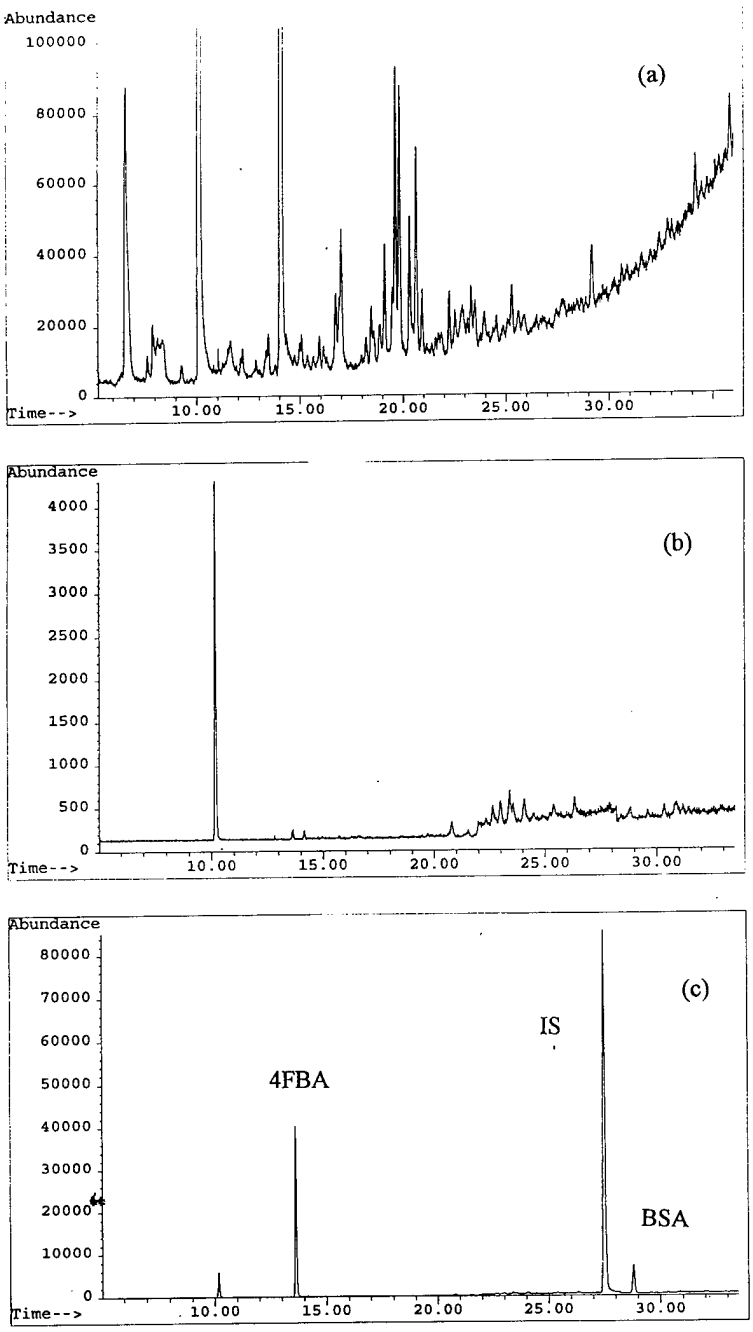

Figure 5. Full scan chromatogram of unspiked gasoline-contaminated groundwater (a), a reconstructed ion chromatogram of unspiked gasoline-contaminated groundwater (b), a reconstructed ion chromatogram of gasoline-contaminated groundwater spiked to give a final concentration of 10 μg/L BSA and 20 μg/L 4FBA.

Table 1. Average recovery of 10 μg/L benzylsuccinic acid (BSA) and 20 μg/L 4-fluorobenzoic acid (4FBA) from 250 mL samples of deionized water, uncontaminated groundwater, and gasoline-contaminated ground water.[a,b]

Sample matrix	Absolute BSA Recovery (%)	Absolute 4FBA Recovery (%)	Relative BSA Recovery (%)[c]
Deionized water	86.2 ± 1.7 (2.0%)	86.5 ± 1.1 (1.3%)	98.2 ± 2.8 (2.9%)
Uncontaminated groundwater (Well 2)	89.4 ± 1.1 (1.3%)	87.6 ± 1.6 (1.8%)	100.6 ± 0.5 (0.5%)
Gasoline-contaminated groundwater (Well 4)	92.2 ± 1.1 (1.2%)	97.2 ± 3.1 (3.2%)	95.1±2.2 (2.3%)

[a] Average recovery of five replicate samples
[b] The relative standard deviation (%) is given in parentheses
[c] Recovery relative to 4FBA

The recovery of BSA and 4FBA from five replicate samples of groundwater collected from Monitoring Well 2 that did not contain BSA nor BTEX above detection was 89.4 ± 1.1% (1.3% RSD) and 87.6 ± 1.6% (1.8% RSD), respectively (Table 1). In addition, a relative BSA recovery of 100.6 ± 0.5 (0.5% RSD) was obtained (Table 1). The recovery of BSA and 4FBA from the gasoline-contaminated groundwater sample collected from Monitoring Well 4 were 92.2 ± 1.1% (1.2% RSD) and 97.2 ± 3.1% (3.2% RSD), respectively, and the recovery of BSA relative to 4FBA was 95.1 ± 2.2% (2.3% RSD) (Table 1). The good precision, indicated by the RSDs <5%, found for the gasoline-contaminated ground water sample, is attributed to the

selective isolation of BSA and 4FBA and the minimal sample handling associated with the method.

Although the detection limit of the complete solid phase extraction with in-vial elution process was not determined, the instrumental detection limit, defined as signal-to-noise ratio (S/N) of 3, is 0.05 μg in the final 1 mL extract. The instrumental quantitation limit for the method, defined as a S/N of 10, is 0.2 μg in the final 1 mL extract.

PART II:

BSA TRANSPORT IN GROUNDWATER

INTRODUCTION

An effective alternative to costly and time-consuming pump and treat methods for remediation of BTEX contaminated aquifers may be *in situ* biodegradation. However, few methods are available to measure in-situ biodegradation rates of BTEX compounds. *In-situ* tests have several advantages over laboratory tests. They preserve the complex chemical, hydraulic, and biological properties of the subsurface. They do not require removal of sediment cores, which typically are quite expensive to obtain and may lead to microbial contamination of the sediment core. In addition, sediment cores are typically small and potentially unrepresentative of larger aquifer volumes. *In-situ* tests can be designed to encompass a large aquifer volume, therefore, addressing some issues of scale. Best of all, they eliminate the extrapolation of laboratory results to field conditions or reproducing field conditions in an artificial laboratory environment.

The single-well "push-pull" test developed by Istok et al. (28) has unique advantages over other *in situ* test methods. Push-pull tests utilize a single well, which avoids the tremendous costs associated with drilling new wells by allowing for the exploitation of existing monitoring wells at a field site. The push-pull tests are simple to perform and can be accomplished in a single day. Mass balance for reactants and products can be obtained during push-pull tests, from which chemical reaction rates can be determined.

Push-Pull Test. A push-pull test consists of the controlled injection of a prepared test solution into a single monitoring well followed by the recovery of the test solution/ground water

mixture from the same well. The test solution contains a tracer and the analyte(s) selected to investigate specific chemical or microbiological activities or aquifer hydraulic properties. There are three main phases of a push-pull test. During the injection ("push") phase, a test solution is introduced into the saturated zone of an aquifer through the screen of an existing monitoring well (Figure 6a). The test solution flows radially outward and penetrates an approximately cylindrical volume of aquifer material centered about the well. The rest or reaction phase, which is optional depending on the goal of the experiment, requires the well to remain undisturbed for a period of time after injection. During the rest phase, the test solution is influenced by natural flow gradients and the indigenous microbial community. During the extraction ("pull") phase, a pre-determined volume of the test solution/ground water mixture is pumped from the well. Because the direction of flow is reversed, groundwater flows radially inward toward the well (Figure 6b). Samples of the extracted water can be analyzed in order to construct breakthrough curves that relate concentration changes of analyte (e.g., BSA) relative to bromide (28). The injected bromide ion serves as a conservative (unreactive) tracer, which moves at the rate of groundwater flow. Changes in the physical flow field that affect solute behavior can be compensated for by comparing solute behavior to that of bromide.

Well Selection Criteria. Since benzylsuccinic acid is produced from the biodegradation of toluene under anaerobic conditions (21), it was desired to investigate the transport and biological stability of BSA under actual groundwater conditions. In order to decouple transport effects from biological effects, it was necessary first to investigate transport of BSA in a uncontaminated well. Monitoring wells at Oregon State University's motorpool facility provided convenient access to a gasoline-contaminated aquifer. As previously described, the OSU

motorpool is the site of a leaking underground fuel storage tank that was removed several years ago.

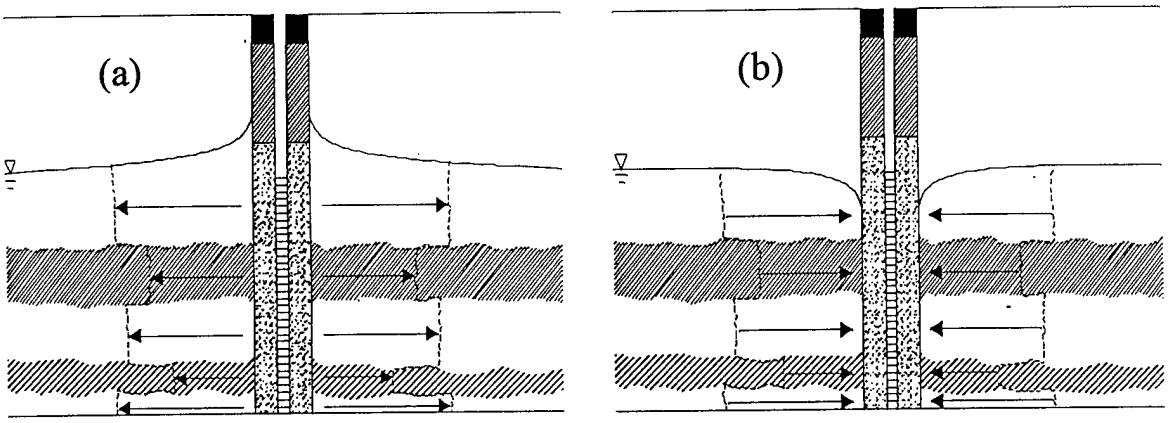

Figure 6. Single-well push-pull test in an unconfined aquifer: (a) injection phase and (b) extraction phase.

Several constraints determined which wells could be used for the BSA transport studies at this site. Only Monitoring Well 4 was in the immediate vicinity of where the gasoline tank had leaked. Six other wells, located several meters away from the gasoline-contaminated area were tested to determine if they had adequate hydraulic conductivity to support a one-day push-pull test. Two wells had sufficient conductivity; however, one was being used by several other research groups. Only Monitoring Well 2 met the hydraulic conductivity requirement and was located sufficiently far from the source of contamination so that it was not contamined with gasoline. Therefore, Monitoring Well 2 and 4 were selected for use in field push-pull tests. General characteristics of the water chemistry were described previously.

EXPERIMENTAL METHODS

Push-Pull Tests. The first push-pull test was conducted in Monitoring Well 2, which was not contaminated by gasoline. For this test, 7.5 g of KBr and 0.5 g of BSA were dissolved into 1L of deionized water then added to a plastic carboy (50 L nominal volume). Deionized water was added and mixed after every 10 L until a final volume of 50 L was reached. The measured bromide and BSA concentrations were 107 and 11.8 mg/L, respectively.

Analytical Methods. Bromide concentrations were determined using a Dionex Model DX-120 ion chromatograph equipped with electrical conductivity detector (Sunnyvale, CA). BSA concentrations were determined using the solid phase extraction with in-vial elution method described earlier. Dissolved oxygen concentrations were determined in the field using a CHEMets (CHEMetrics, Inc., Calverton, VA) colorimetric assay based on Rhodazine D reduction. Nitrate concentrations also were determined by a CHEMets colorimetric assay based on cadmium reduction and organic dye formation.

The test began by measuring the equilibrium water level (1.03 m below land surface), purging the well of approximately 20 liters of water, and collecting the next 4 L of water in order to determine background concentrations of BSA. No BSA was detected in the background groundwater and the dissolved oxygen concentration was 0.5 mg/L. The solution was injected below the water table by gravity drain through nylon-braided tubing at an average flow rate of ~ 0.4 L/min. The water level in the well rose to within 1 cm of the top. On several occasions, it was necessary to stop injection in order to allow the water in the well casing to drop. During each of four waiting periods, triplicate samples of the injectate were collected in 40 mL vials and

capped with Teflon-lined lids. Dissolved oxygen concentration in the injectate was determined to be 4 - 5 mg/L. The test solution was allowed to rest for 1 hr prior to the extraction phase.

During the extraction phase, 150 L of test solution/ground water mixture were extracted at a nearly continuous average rate of ~ 0.6 L/min using a peristaltic pump and the same tubing used for the injection. Triplicate samples were collected in 40 mL vials after every 3 L extraction volume and later analyzed for bromide and BSA. All samples were stored at 4°C until analysis.

The second push-pull test was performed in gasoline-contaminated Monitoring Well 4. Although the overall experiment was performed in a manner similar to the previous push-pull test in Monitoring Well 2, several experimental design changes were made to meet the objective of investigating BSA behavior under anaerobic conditions. Most notable of the changes include the use of nitrogen to dearate the injectate and the use of inflatable "packers" to isolate a section of the well screen. The purpose of the inflatable packers was to restrict the test solution to a short vertical section of the well, thereby obtaining a greater radial zone of influence around the well with an equal volume of test solution. The injectate for the second push-pull test was similar to that used for the first push-pull test with the exception that 0.4 g of BSA was added; the measured bromide and BSA concentrations in the injectate were 104 and 6.8 mg/L, respectively. The injectate was purged with nitrogen for 3 hours prior to and continuously during the injection phase in order to remove oxygen. The initial equilibrium water level in Monitoring Well 4 was 1.77 m below ground surface. The inflatable "packer" assembly, consisting of two inflatable rubber tubes located one meter apart, was installed with the bottom of the packer assembly located 1.3 m above the bottom of the well. The nylon-braided tube for test solution

injection and ground water extraction passes through the upper inflatable tube and ends midway between the two rubber tubes. Moderate air pressure (1.3 atm) was used to inflate the tubes, thereby isolating a 1 m portion of the well. All water during this test was injected or extracted from within this 1 m zone, including the initial 24 L purge volume and 4 L background sample.

The test solution was injected by gravity-drain at an average flow rate of ~ 0.2 L/min. Due to losses during sampling and problems with equipment, 8.5 L of test solution were not injected. Water level in the well rose to 0.6 m by the end of the injection phase. Background and injectate samples were collected as before, dissolved oxygen concentration was 0.7 mg/L in the injectate samples. The concentration of BSA in the background samples was 2.2 μg/L; however insufficient mass was available to confirm the identification of BSA using full scan conditions on the GC/MS. Due to the slower injection rate (half that of the first push-pull test) and problems with equipment, the test solution was left in the aquifer overnight and pumped out the next morning, resulting in a 16 hour rest phase.

During the extraction phase, 104 L of test solution/ground water mixture were extracted at an average rate of ~ 0.2 L/min using a peristaltic pump and the same tubing used for the injection. Water level in the well dropped to 3.2 m by the end of the extraction phase. Triplicate samples were collected in 40 mL vials after every 2 L extraction volume and later analyzed for bromide and BSA. All samples were stored at 4°C until analysis.

RESULTS AND DISCUSSION

First Push-Pull Test, Uncontaminated Monitoring Well 2. Breakthrough curves were constructed to display relative concentrations (C/C_o) versus the volume extracted/volume

injected, where C is the measured solute (bromide or BSA) concentration in a sample, and C_o is the measured solute concentration in the injected test solution (Figure 7a). Since bromide is a conservative (nonreactive) tracer, it is subject to the same dispersive and advective forces influencing the flow of ground water. Therefore, as the injected plume moves away from the well some mixing with native ground water occurs. When extraction begins, the first water recovered is essentially the test solution because it has not flowed very far from the well, recall the rest phase was only 1 hour. As time proceeds, the extracted water consists of a mixture of test solution diluted by ground water. As a result, the extraction phase breakthrough curves for bromide begin with C/C_o close to unity and rapidly decrease toward zero with a relatively long tail as very dilute test solution is extracted (Figure 7a). The cause of the sharp dip in the breakthrough curve is unknown. Inspection of field data indicates that water level had returned to the initial equilibrium level before extraction began and that pumping rates were essentially constant during this portion of the extraction.

The mass of solute recovered (m) relative to the total mass of solute injected (m_o) was plotted versus the volume extracted/volume injected in order to obtain mass recovery plots (Figure 7b). The total mass of each solute recovered was obtained by numerically integrating the area under each breakthrough curve. Bromide mass recovery in this experiment rose quickly in the beginning and slowly approached a constant value corresponding to a total mass recovery of 52% (Figure 7b). There is a reasonable qualitative explanation for such low bromide recovery. The test solution was injected and extracted over the entire length of well screen (~ 3.5 m in total length). During injection, water levels were allowed to rise to within 1 cm of the top of the well casing. This mounding was desired at the time to develop a large hydraulic gradient that would

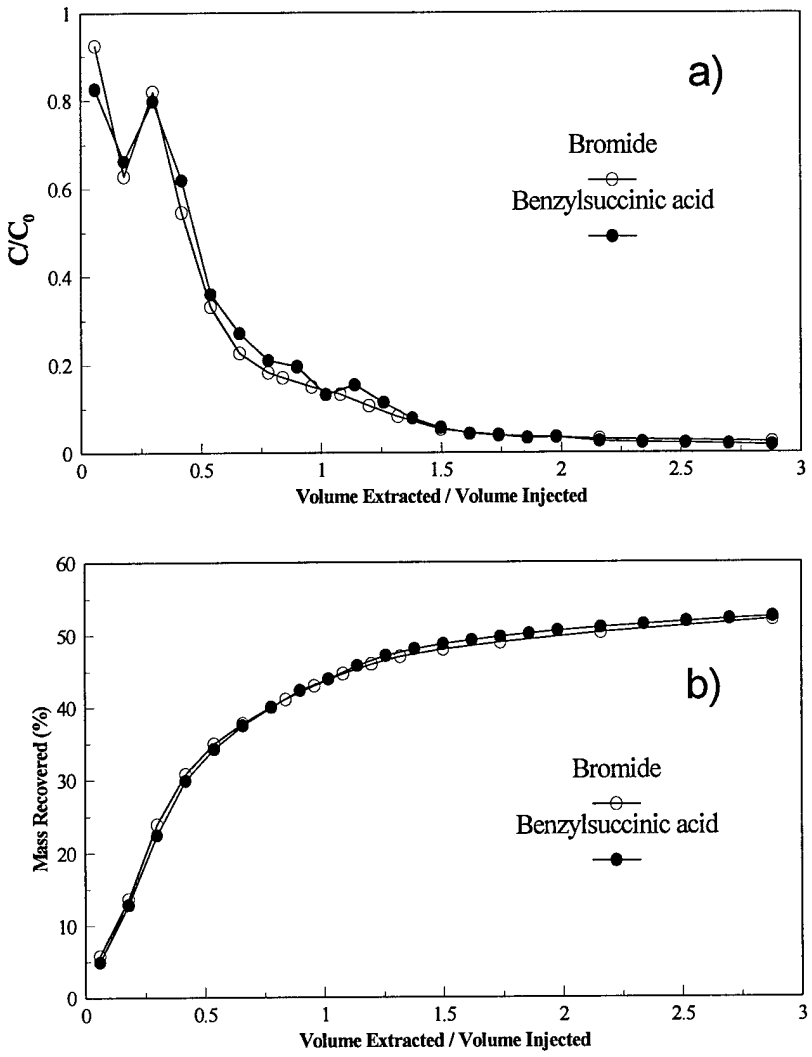

Figure 7. Extraction phase breakthrough curves (a) and mass recovery plots (b) for bromide and BSA during a field push-pull test conducted in uncontaminated Monitoring Well 2.

drive the test solution into the aquifer over a short time period so that the push-pull test could be completed in one day. As a result, the test solution probably flowed into portions of the aquifer above the water table (as much as 0.5 m above) and may have traveled a significant distance

during the one hour rest phase. Just the opposite effect occurred during extraction as water levels were allowed to drop to 3.3 m, which is 2.3 m below the equilibrium level and only 0.7 m above the bottom of the well. As a consequence, a large fraction of the water extracted from the well, may have contained a small fraction of the test solution, which resulted in dilution of the extracted test solution with ground water. Extracting three times the injection volume should have recovered ~ 90% of the injected bromide, however, in this case, a significant fraction of test solution remained in the aquifer.

The extraction phase breakthrough curve for BSA (Figure 7a) closely matches that of bromide, indicating that BSA also was transported conservatively during this test. We did not expect BSA to be retarded due to its di-carboxylic acid functional groups which are likely to be either partially or fully deprotonated at the pH of the ground water in this well (pH of 7.0), as a result, BSA would exhibit minimal attraction for the negatively-charged sediment.

Although only 52% of bromide mass was recovered, BSA mass recovery tightly tracked that of bromide (Figure 7b), which supports the breakthrough curve data. Conservative behavior of BSA observed in the breakthrough and mass recovery curves indicate that BSA transport is conservative and BSA is biologically stable under the conditions of this 8 hour test.

The notion that the injectate was diluted significantly with ground water is supported by the trend in dissolved oxygen concentration. Dissolved oxygen concentration measured ~ 6 mg/L in the first samples recovered from the well during the extraction phase and steadily decreased to ~ 1 mg/L after extracting 48 L of water, corresponding to a volume extracted/volume injected ratio of 1.1 (Figure 7a). The decline is consistent with the injectate having 4-5 mg/L dissolved oxygen while the background dissolved oxygen was 0.5 mg/L. Due to problems with sampling

equipment, air bubbles were observed in the pump-discharge tubing and dissolved oxygen measurements below 1 mg/L were unattainable during the remainder of the test, the actual concentration probably dropped to < 1 mg/L.

Second Push-Pull Test, Gasoline-Contaminated Monitoring Well 4. The injection flow rate and extraction flow rate were reduced to one-half and one-third, respectively, of the flow rates used in the first test in an attempt to avoid the suspected dilution problem that potentially lead to the low mass recovery obtained in the uncontaminated well push-pull test. A target of 0.5 m was set for the maximum allowable water-table buildup or drawdown during this test and inflatable "packers" were used to try and minimize dilution by releasing the injectate into a smaller vertical zone. However, due to low hydraulic conductivity two days were required to complete the test and water levels rose as much as 1.1 m during injection and drawdown was as much as 1.5 m during extraction. The excessive deviations in water level from the equilibrium value and the 16 hour rest phase may have again caused significant dilution of the injectate, resulting in low mass recovery of bromide and BSA.

The extraction phase breakthrough curve for bromide in this test (Figure 8a) followed a similar trend as that of the first push-pull test (Figure 7a), except that bromide C/C_o values initially rise, indicating the center of mass of the injected test solution had migrated away from the well during the 16 hour rest phase.

The use of the extended 16 hour rest phase caused the slope of the bromide mass recovery curve (Figure 8b) to be somewhat less steep than that of the mass recovery curve associated with the 1 hour rest phase in the first push-pull test (Figure 7b). Note that bromide mass recovery is still rising after extracting two and a half times the injected volume and only 48% was recovered.

This is probably the combined result of a long rest phase and the less than ideal water-table buildup and drawdown during the injection and extraction phases of this test, which may have led to significant dilution of the test solution, as described previously.

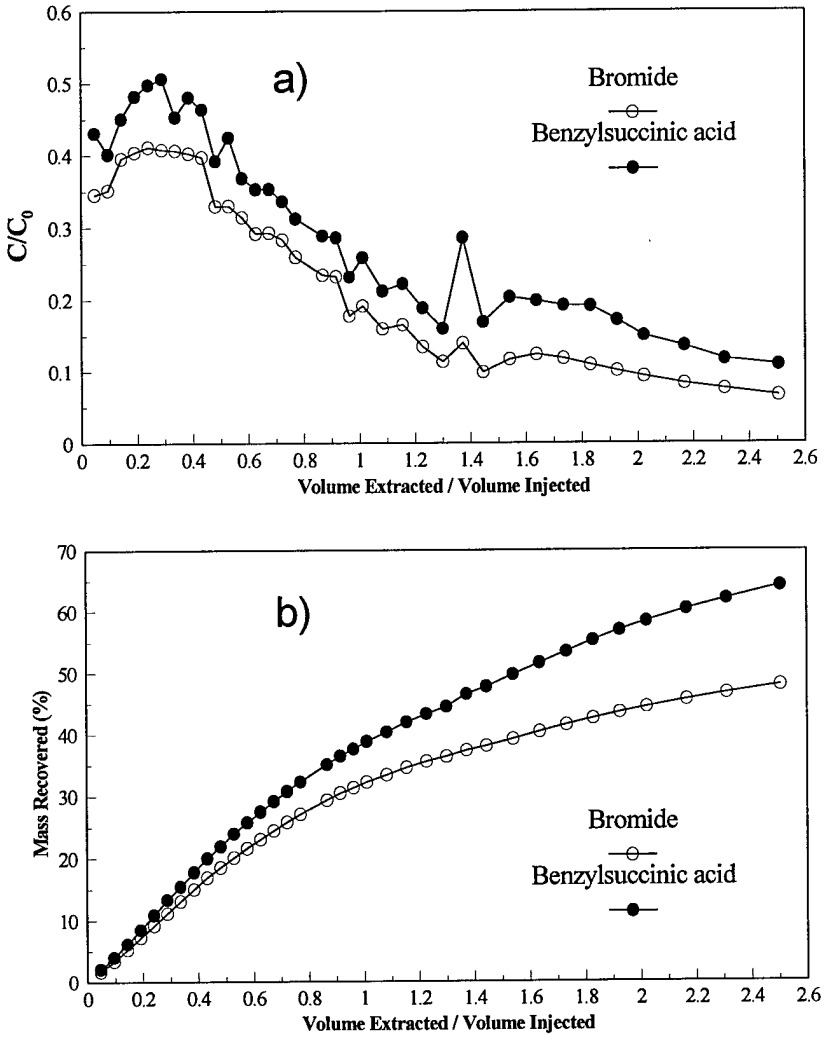

Figure 8. Extraction phase breakthrough curves (a) and mass recovery plots (b) for bromide and BSA during a field push-pull test conducted in gasoline-contaminated Monitoring Well 4.

The extraction phase breakthrough curve for BSA mimics that of bromide (Figure 8a); however, C/C_o values of BSA are higher than those of bromide. Notice that many of the dips and peaks in the BSA breakthrough curve are coincident with similar features in the bromide breakthrough curve. This indicates that the variations in BSA concentrations may be caused by the same physical phenomena as those affecting bromide concentrations; however, does not explain the higher C/C_o values for BSA relative to bromide. Subtracting background levels of BSA (2.2 μg/L) had a negligible effect on lowering the values of C/C_o for BSA. Since no obvious physical reason can be determined to explain the abnormally high BSA C/C_o values, one must consider possible analytical reasons. A high C/C_o ratio is caused by either a high BSA concentration in the sample or a low injectate BSA concentration, or both. The BSA injectate concentration is somewhat suspect because it was determined from the upper portion of the analytical calibration curve, which may have lead to erroneous quantitation results. This hypothesis is supported by the fact that measured BSA concentration in the five samples of injectate were only 6.8 mg/L; whereas, the expected injectate concentration calculated from the 0.4 μg of BSA that was added to 50 L of water is 8.0 mg/L.

The BSA mass recovery is shown to be greater than that of bromide (Figure 8b). Although this is easily deduced from inspection of the BSA breakthrough curve (Figure 8a), it should not be the case. Since bromide is an unretained tracer compound, it is expected that all other solutes will have a mass recovery equal to or less than that of bromide, after accounting for any background concentrations.

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